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Uptake and Transport of Superparamagnetic Iron Oxide Nanoparticles through Human Brain Capillary Endothelial Cells

L. B. Thomsen,*[,†] T. Linemann,[†] K. M. Pondman,[‡] J. Lichota,[†] K. S. Kim,[§] R. J. Pieters,[∥] G. M. Visser,[⊥] and T. Moos[†]

[†]Department of Health Science and Technology, Aalborg University, Aalborg, Denmark

[‡]Neuroimaging group, MIRA institute, University of Twente, Enschede, The Netherlands

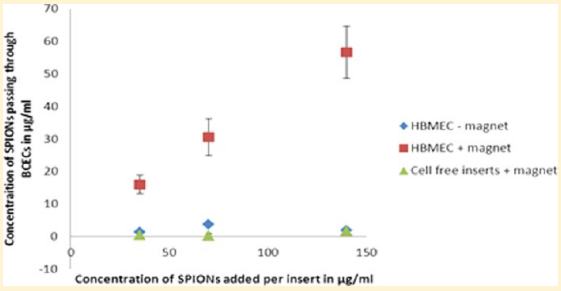
[§]Division of Pediatric Infectious Diseases, John Hopkins University, School of Medicine, Baltimore, Maryland 21218, United States

[∥]Department of Medical Chemistry and Chemical Biology, Utrecht University, Utrecht, The Netherlands

[⊥]Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands

Supporting Information

ABSTRACT: The blood–brain barrier (BBB) formed by brain capillary endothelial cells (BCECs) constitutes a firm physical, chemical, and immunological barrier, making the brain accessible to only a few percent of potential drugs intended for treatment inside the central nervous system. With the purpose of overcoming the restraints of the BBB by allowing the transport of drugs, siRNA, or DNA into the brain, a novel approach is to use superparamagnetic iron oxide nanoparticles (SPIONs) as drug carriers. The aim of this study was to investigate the ability of fluorescent SPIONs to pass through human brain microvascular endothelial cells facilitated by an external magnet. The ability of SPIONs to penetrate the barrier was shown to be significantly stronger in the presence of an external magnetic force in an *in vitro* BBB model. Hence, particles added to the luminal side of the *in vitro* BBB model were found in astrocytes cocultured at a remote distance on the abluminal side, indicating that particles were transported through the barrier and taken up by astrocytes. Addition of the SPIONs to the culture medium did not negatively affect the viability of the endothelial cells. The magnetic force-mediated dragging of SPIONs through BCECs may denote a novel mechanism for the delivery of drugs to the brain.



KEYWORDS: *blood–brain barrier, magnetic nanoparticles, drug delivery, TEER, astrocytes, endothelial cells, in vitro*

The delivery of drugs to the brain has proven to be a difficult task mainly because of the presence of the blood–brain barrier (BBB) formed by tightly interconnected brain capillary endothelial cells (BCECs). The impermeability properties of the BCECs are supported by astrocytes, pericytes, and neurons that together form the so-called neurovascular unit.[1] The BBB precludes most molecules from entering the central nervous system (CNS) because of the presence of efflux transporters,[2] and molecules must be preferably small in size and lipophilic to enter the brain. Even molecules with these properties have only limited success in crossing the BBB and entering the brain.[3]

Many different colloidal drug carriers have been created, e.g., liposomes or polyplexes, which fulfill the demands of being at the nanosize scale. These drug carriers have a hydrophilic outward surface that allows them to circulate in blood plasma. Most of these carriers, however, fail to deliver their cargo to the brain in an amount adequate for treatment without allowing unacceptable high off-target delivery. A relatively new approach in the field of drug delivery is the use of magnetic nanoparticles. Magnetic nanoparticles are currently being used for various biomedical purposes such as a contrast agent for magnetic resonance imaging (MRI),[4] induction of hyperthermia for tumor therapy,[5] cell labeling and cell separation,[6,7] targeted therapeutics,[8,9] and magnetofection.[10]

Superparamagnetic iron oxide nanoparticles (SPIONs) constitute a subtype of magnetic nanoparticles that are highly magnetizable and have a core of iron oxide particles composed of magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3).[11] Typically, the SPIONs have a mean diameter of 50–100 nm,[11] and their iron oxide core exerts low toxicity, as it is gradually degraded to Fe^{3+} in the body and enters the pool of body iron;[11] e.g., SPIONs induce oxidative stress only in murine macrophage (J774) cells at doses higher than 100 μg/mL.[12] The magnetic core of SPIONs can be coated with lipophilic fluorescent dyes for visual detection. Furthermore, the particles can be protected by biocompatible polymeric shell materials like dextran, polysorbate, or starch or coated by phospholipids or polyethylene glycol (PEG) to prolong their presence within the circulation because of a lower level of recognition of the

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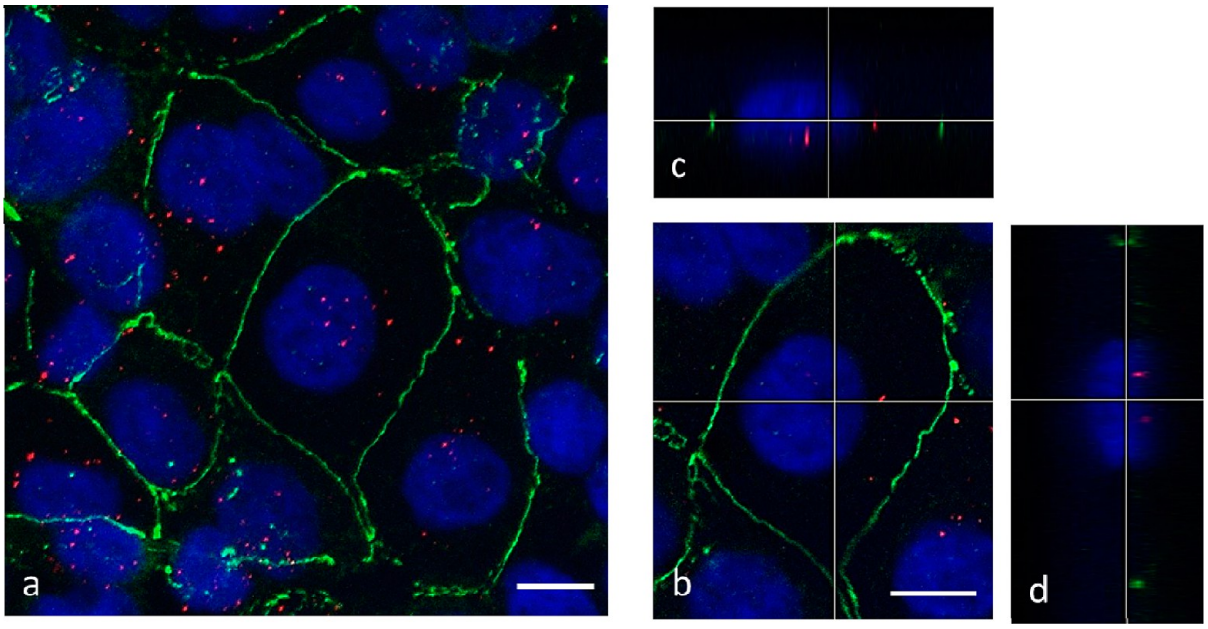

Figure 1. Confocal images of BCECs stained for the tight junction protein ZO-1 (green) and nucleus (blue). SPIONs (140 μg) were added to each insert with BCECs and incubated for 24 h. The SPIONs (red) can be seen scattered around the inside of the cells: (a) a montage of a Z-stack of 25 slides, (b) a single slide in the Z-stack ($X-Y$ plane) with focus on a single cell, (c) an $X-Z$ plane of panel b, and (d) a $Y-Z$ plane of panel b. Notably, SPIONs do not associate with the green color of ZO-1, indicating that they do not appear in the paracellular spaces between the BCECs. Likewise, the $Y-Z$ plane shows that the SPIONs are not distributed to the nucleus. The gray bars in panel b represent the site at which the $X-Z$ (c) and $Y-Z$ (d) sections are shown. The scale bar is 10 μm.

particles by the reticulo endothelial system.[9,13−16] A proper material coat also prevents the particles from aggregating, which they otherwise tend to do, because of a strong magnetic dipole to dipole attraction.[13,17] Furthermore, a protective coat allows conjugation of, e.g., various proteins, DNA, and drugs to the surface of the SPIONs.[13,15−18]

A major advantage of the properties of SPIONs is their ability to be magnetized only in the presence of a magnetic field. A magnet can therefore be used to direct the delivery of the SPIONs to a given target organ.[13,17] Under the influence of the magnetic field, the SPIONs are dragged toward the magnet to concentrate near its location. Delivery of SPIONs will therefore benefit from being very local, and its dosing can be minimized to reduce off-target effects.[13,17] In this study, the ability of SPIONs to cross into and through HBMECs is investigated in an *in vitro* BBB model. The SPIONs pass into HBMECs and increasingly pass the intact brain endothelial cell monolayer with the aid of an external magnet, ending up in a layer of astrocytes cultured at a remote distance on the "brain side" of the endothelial cells.

RESULTS AND DISCUSSION

In this study, we investigate whether SPIONs are able to enter into and across a monolayer of BCECs cultured in an *in vitro* BBB model and if an external magnetic force can be applied to improve the penetration rate and efficiency. We also wanted to test if the SPIONs would exert toxicity on the BCECs and obstruct the barrier when passing the endothelial cell layer.

Size and Charge of the SPIONs. The hydrodynamic diameter of the SPIONs was determined by DLS, which is a back scatter analysis. The SPIONs had a mean diameter of 117.4 ± 0.27 nm [±standard error (SE); n = 5]. Furthermore, the ζ potential of the SPIONs was measured to be −16.9 ± 0.6 mV (±SE; n = 3). The starch-coated SPIONs were therefore determined to have a slightly negative surface charge. Similar starch-coated SPIONs have previously been found to be of similar anionic charge.[19] A negative ζ potential favors less plasma protein adsorption and therefore increases the plasma circulation time of the particles.[11] The particles are therefore suitable for not only *in vitro* application but also *in vivo* application.

SPIONs Enter BCECs. BCECs that had been incubated with SPIONs for 24 h but not exposed to a magnetic force were investigated. The BCECs were stained for the endothelial specific tight junction protein ZO-1 to visualize the cell boundaries, and the nuclei were stained with DAPI (Figure 1). SPIONs were found distributed throughout the BCEC monolayer. In Figure 1, it can be seen that the SPIONs were found inside the cells in different focal planes and within the cell boundaries. This indicates that SPIONs are taken up by the BCECs even without the addition of an external magnetic force. The SPIONs do therefore not need any further chemical or physical changes on their surface to interact with BCECs and subsequently be internalized by the cells. SPIONs were also able to pass the BCEC monolayer at a low concentration without the external magnetic force. No particles passed the cell free inserts that were not subjected to an external magnet, and therefore, the passage of SPIONs through BCECs without the aid of a magnet is most likely due to active, energy-dependent transcellular transport.[20] Studies have shown that the cell−nanoparticle interaction is complicated because most nanoparticles administered both *in vivo* and *in vitro* are covered with proteins and lipids from the plasma and media forming a soft and hard corona on their surfaces.[20,21] This corona is most likely what the "cell sees" and not the nanoparticle itself.[20,21] Therefore, the cell−SPION interaction should be further investigated both *in vivo* and *in vitro* to learn more about how the SPIONS are taken up by the BCECs.

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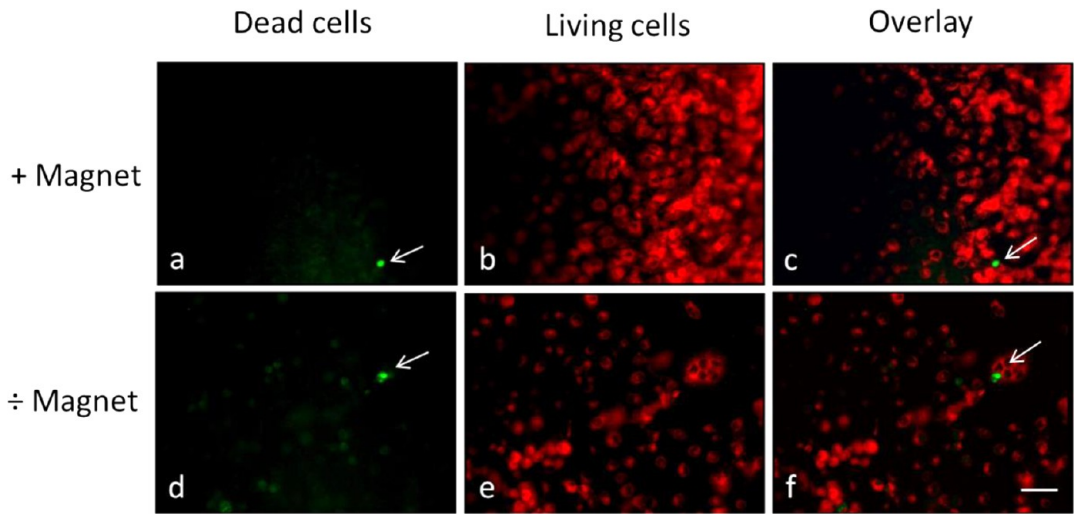

Figure 2. Live−dead cell viability staining of BCECs. The top row shows BCECs in inserts that had been exposed to a magnetic force. The bottom row shows BCECs in inserts that had not been exposed to a magnetic force. SPIONs (70 μg/insert) were added. Dead cells are visualized with Sytox green stain and uptake (a and d) (indicated by white arrows). Live cells are visualized with Resazurin staining (b and e). Overlays of the two stains are seen in panels c and f. There are no differences in viability between the cells with and without exposure to a magnetic force. The scale bar is 50 μm.

Exposure to SPIONs and a Magnetic Force Does Not Lead to Cytotoxicity of the BCECs. SPIONs have been shown to induce oxidative stress,[12] and therefore, we wanted to test the cell viability within the time frame of this study. It is also known that cells of different origin react differently upon exposure to SPIONs; this phenomenon is called the "cell vision".[22,23] It has been shown that brain cells are quite sensitive to SPIONs compared with, e.g., liver and heart cells.[22,23] Furthermore, there was speculation about whether the dragging of SPIONs by a magnetic force through the BCEC monolayer would cause damage to the cells. The cell viability was tested with a live−dead viability assay. Our study revealed no signs of lost viability of the BCECs after they had been incubated for 24 h with various concentrations of SPIONs (35, 70, and 140 μg/mL) (Figure 2). A Trypan Blue stain experiment conducted to count the amount of dead cells in wells incubated for 24 h with or without 140 μg/insert and exposed to a magnetic force for 5 h revealed no statistical difference between cell viability under the two conditions ($p < 0.05$).

SPIONs in general have been shown to exhibit a generally low but concentration-dependent cytotoxicity mostly due to the iron core.[11,12] Naqvi et al. observed that the toxicity of SPIONs that are 30 nm in diameter with a Tween 80 coat increases in a concentration-dependent manner.[12] In their measurements, the toxicity seen as a marked change in cell viability was observed when between 100 and 200 μg/mL SPIONs were added to cultures of murine macrophage cells (J774), indicating that SPIONs are nontoxic to cells at concentrations of ≤100 μg/mL.[12] These data are in good accord with the results of this study even though the concentration of 140 μg/mL lies within their range of a toxic concentration but does not exhibit any toxic effect on cells in our study after incubation for 24 h and exposure to a magnetic force for 5 h.

In this study, the cytotoxic effect was tested within the time frame of 29 h. If SPIONs are to be administered *in vivo* within a broader time frame, the cytotoxic effect needs to be further examined. It has, though, been shown that SPIONs 50−150 nm in diameter with an anhydroglucose polymer coat did not affect the mortality of Sprague-Dawley rats when the SPIONs were injected into the tail veins in a dose of 5% of the estimated blood volume.[24] The rats were monitored for up to 65 days, and it was detected that the amount of magnetic particles found in the animal decreased over time.[24] Together, this indicates that magnetic nanoparticles can probably be administered systemically without exerting toxicity on animals. It will also seem evident that when given *in vivo* the particles will be cleared and probably be deposited as iron stores inside cells of the brain, most likely in ferritin that forms a major repository for iron in both neurons and glia capable of binding approximately 4500 atoms of iron per ferritin molecule.[25,26]

Integrity of the BCECs Forming an *in Vitro* BBB. The effect of SPIONs was further investigated by monitoring the integrity of the BCECs before and after application of SPIONs followed by exposure to a magnetic force. The average maximal TEER value of BCECs that were not subjected to either SPIONs or the magnetic force was on average 43.2 ± 0.49 Ω cm^2 (±SE). Before the experiment, the TEER value was on average 43.3 ± 0.44 Ω cm^2 (±SE). The TEER value after addition of SPIONs and incubation for 5 h without exposure to the magnetic force was on average 43.7 ± 0.22 Ω cm^2 (±SE). The average TEER value after addition of SPIONs following a 5 h exposure to the magnetic force was 43.9 ± 0.32 Ω cm^2 (±SE).

The results show that the TEER values did not decrease after a 24 h incubation with SPIONs or a 24 h incubation with SPIONs followed by a 5 h incubation on a plate magnet. The stable TEER values before and after exposure to the SPIONs and magnetic field indicate that the integrity of this *in vitro* BBB was not harmed by the magnetic field-aided penetration of the SPIONs.

This observation is in good agreement with the findings of Saiyed et al., who showed that magnetic particles were encapsulated in liposomes, taken up by monocytes, and drawn through an *in vitro* BBB model with an external magnet without affecting TEER values.[17] Furthermore, an *in vivo* study has shown that paravascular passage of polystyrene-entrapped magnetic nanoparticles (124 nm) did not seem to affect the integrity of the BBB in 10-week-old mice.[27]

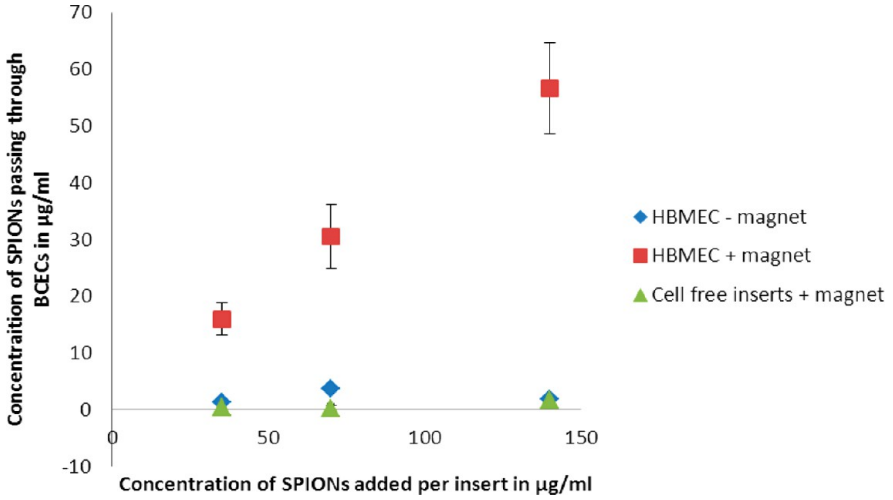

Figure 3. Quantitative count between the concentrations of SPIONs added to inserts and their passage through BCECs in inserts and into wells containing astrocytes. Inserts with BCECs (red) and cell free inserts (green) that were both subjected to an external electric field for 5 h and inserts with BCECs (blue) devoid of exposure to an external electric force. SPIONs were added to BCECs at a concentration of 35, 70, or 140 μg/insert. The amount of SPIONs passing the BCECs and entering into astrocytes was clearly larger when an external magnetic field was applied ($p < 0.001$ at either concentration added). There seems to be a linear correlation between the dose and response upon application of the magnetic field [$n = 4$ observations per point at 70 and 140 μg, and $n = 3$ observations per point at 35 μg (results are means ± SE)].

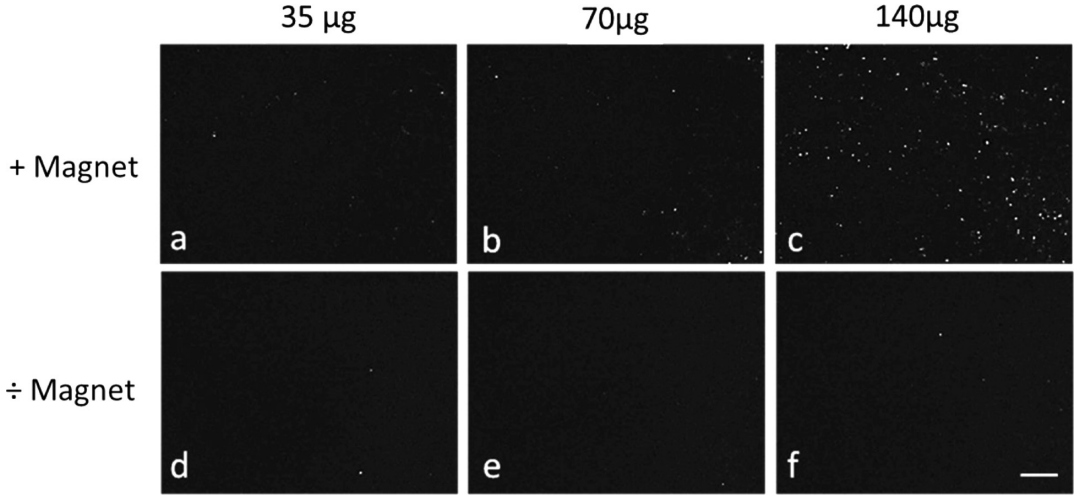

Figure 4. Pictures taken of different wells in the presence of SPIONs (white dots) that have passed initially from the luminal chambers of inserts containing BCEC monolayers and into wells placed underneath the inserts. The SPIONs were added to the inserts in amounts of 35 μg (a and d), 70 μg (b and e), and 140 μg (c and f). Together, the inserts and wells were either exposed to a magnetic field for 5 h (top) or incubated for 5 h without being exposed to a magnetic field (bottom). The inserts was removed from the wells and the wells checked for the presence of SPIONs. The concentration of nanoparticles was visually higher when the external magnetic field was applied (top). For better visualization, the red fluorescent SPIONs are colored white. The scale bar is 30 μm.

The TEER values of the BCECs in this study are rather low, but this is not unusual for brain microvascular cell lines.[28−30] TEER depends on how tightly BCECs are interconnected via tight junctions, and a low TEER could theoretically indicate that there are open areas between BCECs.[28] An immunostaining was performed on the BCECs to visualize the state of the tight junctions. BCECs stained positive for the tight junction protein ZO-1, showing the presence of tight junctions between the cells (Figure 1), but some inconsistencies were observed in terms of less prominent ZO-1 immunoreactivity. Therefore, precautions were taken to avoid paracellular transport of SPIONs through the irregular tight junctions of the BCECs. Hence, a washing step was applied on the cell free inserts and inserts containing BCECs after incubation with SPIONs for 24 h to remove excess particles (Figure 6). After the washing step, half of the cell free inserts and inserts on the experimental plates were subjected to a magnetic force for 5 h. No particles were detected under the cell free inserts that had not been subjected to the magnetic force. Only very few SPIONs passed the membrane in the cell free inserts that were washed and subsequently subjected to the magnetic force for 5 h and in an amount that was significantly ($p < 0.01$) smaller than the amount passing through BCECs that can be seen in Figure 3. This clearly indicates that the washing step ensured a virtually complete depletion of excess SPIONs in the medium at the luminal side and that SPIONs pass through BCECs by means of transcellular transport and not paracellularly.

Passage of SPIONs through the BCECs *in Vitro*. The SPIONs crossed the thin BCEC monolayer under the influence of the external magnet placed underneath the culture plates,

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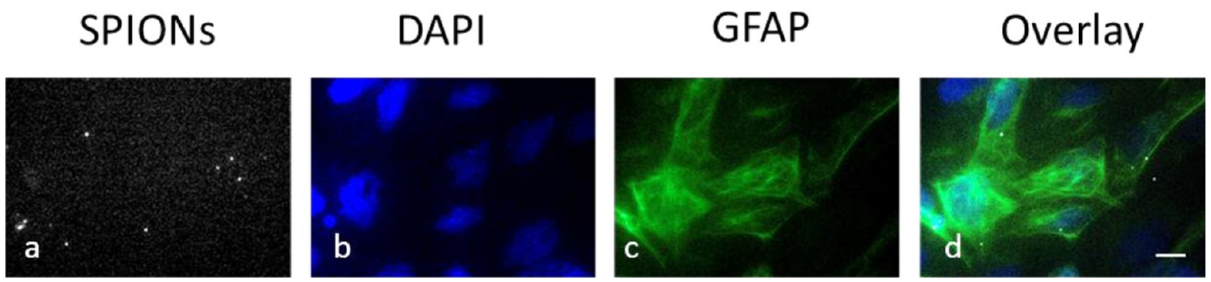

Figure 5. Fluorescence microscopy showing SPIONs (a) inside DAPI-stained (b) and GFAP-stained (c) astrocytes present at the bottom of a lower well of a culture plate. An overlay of panels a–c is shown in panel d. The astrocytes are from a plate that had been exposed to a magnetic field for 5 h. The pictures show the presence of fluorescent SPIONs inside astrocytes cultured in a plate well under an insert to which 70 μg of SPIONs had been added. The red fluorescent SPIONs are colored white for better visualization. The scale bar is 15 μm.

and their passage occurred in a concentration-dependent manner (Figure 4a–c). A limited amount of SPIONs was observed in the lower chamber without exposure to the magnetic field (Figure 4d–f), and this amount did not seem to increase with an increase in the concentration of the nanoparticles added. The amount of particles that penetrated the BCECs and entered into the wells on the abluminal side was additionally quantified in the control and experimental plates (Figure 3). There was a statistically significant difference between the concentrations of SPIONs detected in wells subjected to the magnetic force and wells that were not subjected to the magnetic force ($p < 0.001$ for 35 μg, $p < 0.001$ for 70 μg, and $p < 0.001$ for 140 μg). The concentrations of SPIONs passing the BCEC monolayer were increased 11-, 8-, and 29-fold by the magnetic force at concentrations of 35, 70, and 140 μg/mL, respectively.

As previously described, a small amount of particles passed the membrane in the cell free inserts that had been subjected to the magnetic field. No particles were found to pass the membrane in the cell free inserts that had not been subjected to the magnetic field. There was a significant difference between the amount of SPIONs passing the BCECs and the amount of SPIONs passing the cell free inserts when both had been subjected to the external magnetic force ($p < 0.001$ for 35 μg, $p < 0.001$ for 70 μg, and $p < 0.001$ for 140 μg). The amounts of SPIONs passing BCECs when they were exposed to a magnetic force were 32–120 times larger than the amounts passing cell free inserts that had also been subjected to a magnetic force.

The results show that significant amounts of SPIONs passed the BCECs when they were subjected to the external magnetic force (Figure 3). Chertok et al. observed that a magnetic force of 0.4 T increased the concentration of starch-coated SPIONs (~110 nm) targeted toward a rat brain tumor by 11.5-fold over the concentration found in nontargeted (no magnetic force applied) brain tumors.[13] Similar results have been shown for starch-coated SPIONs with a diameter of 46 nm that were intravenously injected into nude mice with tumor xenografts.[31,32] The SPIONs were shown to accumulate at a higher concentration in the tumors when they were subjected to an external magnetic field of 0.5 T.[31,32] These studies all refer to magnetic force-increased delivery of SPIONs in tumor tissue that is known to have a compromised blood–tumor barrier. Chertok et al. found that the concentration of SPIONs dispersed into normal brain tissue of Fischer 344 rats seemed to increase slightly (~3-fold) under the influence of a magnetic field (0.4 T) versus that of non-magnetic-force-targeted SPIONs.[13] Also, the migration of monocytes loaded with magnetic liposomes has been shown to be enhanced 3-fold via application of a magnetic force in an *in vitro* BBB model.[17] The amount of polystyrene-entrapped magnetic nanoparticles found in the brain tissue of 10-week-old mice after systemic delivery was increased by 25-fold with the aid of an external magnet with a strength of ~1 T.[27] In this study, the extent of passage of SPIONs across an *in vitro* BBB was clearly increased manyfold by a magnetic force. The rate of penetration of SPIONs into BCECs without any aid of an external magnetic field was low and did not significantly change in spite of a change in the concentration of the dose. The concentration of SPIONs added to the inserts containing BCECs linearly correlated with the concentration entering the astrocytes cultured in wells that had been subjected to an external magnetic field. Hence, these results support the strategy of employing SPIONs for targeted delivery to the brain in general even in neurologic disorders without a compromised BBB and not only in brain tumors where the BBB is defective.

SPIONs Pass through a BCEC Monolayer and Further into Astrocytes. The ability of SPIONs to cross a BCEC monolayer and to enter cells on the brain side was also investigated. Astrocytes were cultured on the bottom of the wells, but otherwise, the experimental setup was the same as in the previous sections with BCECs cultured in inserts inserted into wells containing astrocytes. The astrocytes were stained with GFAP and DAPI. Fluorescent SPIONs were found in astrocytes in culture plates underneath inserts with BCECs that had been exposed to a magnetic force (Figure 5) as well as culture plates underneath inserts with BCECs that had not been exposed to a magnetic force. However, only a minimal amount of particles was observed in astrocytes when no magnetic force was applied as compared to that of astrocytes that had been subjected to a magnetic force. This suggests that the SPIONs not only are drawn through the BCEC monolayer but also enter cells present a remote distance from the abluminal side of the BCECs. Chertok et al. observed the presence of intravenously injected SPIONs in the brain parenchyma of normal rat brain tissue.[13] Hence, a possible application of the SPIONs for drug delivery seems applicable not only to BCECs but also to the neurons and glial cells located deeper inside the brain, as SPIONs passing though the BCECs are likely to be taken up by these cells, too. This notion, together with the fact that the SPIONs are capable of movement in a particular direction via the application of an external magnetic field, signifies these magnetizable particles as potential drug carriers. The SPIONs are therefore candidates for being drug carriers for CNS drug delivery beyond the BBB. Interestingly, SPIONs very similar to those used in this study ~110 nm in diameter and coated with starch and were shown

to enter the brain of Fischer 344 rats in rather small amounts when injected intravenously without the presence of a magnetic force.[13] Application of a magnetic force applied externally to the skull may therefore be an interesting mechanism for ensuring local and improved delivery of SPIONs to the brain that warrants further examination.

The *in vivo* BBB consists of three cell types in different layers. The first is BCECs surrounded by pericytes and covered by astrocytes.[1] Penetration of the *in vivo* BBB after intravenous injection of SPIONs will probably require a magnet stronger than the one used in this study, not only because of the thickness of the barrier but also because of a greater distance between the capillaries and the magnet if the magnet is placed externally on the scull. Furthermore, the drag of blood flow will exert a secondary force on the particles. Furlani et al. described a model that included the drag of blood flow accounting for the hematocrit of the blood, and a magnet for the capture of particles in magnetic drug delivery.[33] Using a cylindrical magnet with a diameter of 6 cm at a position 2 cm from the target location will allow trapping of the particles at the vessel wall and exerts a magnetic force on the particles of 9.6×10^{-18} N (see the Supporting Information), which is larger by a factor 1000 than the magnetic force applied in this *in vitro* study, which was capable of pulling the particles though the BBB.[33] The model in this study cannot take into account all factors of the *in vivo* situation; therefore, passage of SPIONs through the *in vivo* BBB should be investigated.

CONCLUSIONS

SPIONs pass into and through a BCEC monolayer and enter astrocytes cultured at the bottom of lower chambers in a manner that is significantly enhanced by the use of an external magnetic force. The external magnetic force does not affect the integrity of the endothelial monolayer, nor is the cell viability affected by the fluorescent SPIONs or by the magnetic force dragging the nanoparticles through the cells. The data in this study support a strategy of using SPIONs for a two-step strategy for the delivery of proteins to the brain. This two-step delivery strategy was proven by Jiang and co-workers, who first transfected cultured mouse brain capillary endothelial cells (MBEC4) with pIRESneo-mGDNF using Lipofectamine and subsequently observed secretion of the GDNF of interest for delivery to the neurons and glia of the brain.[34] SPIONs could be utilized for the delivery of DNA fragments to BCECs, which following transfection might secrete the DNA-encoded proteins into the brain.

If systemically injected, the SPIONs used in this study would probably also interact with endothelial cells in regions other than the brain. Therefore, a targeting strategy directed toward BCECs would be warranted. The SPIONs can be coated with substrates that are able to bind, e.g., ligands or antibodies,[15,16], and a strategy could be to conjugate SPIONs with an anti-transferrin receptor antibody known to enhance uptake by BCECs.[35−37] In conclusion, SPIONs may potentially be used for delivery and transport to BCECs and even further into the brain seemingly without harming the cells.

METHODS

Materials. Transwell membrane culture inserts and plates (Corning, Thermo Fisher Scientific), fluorescent SPIONs "nanoscreenMAG-D" composed of magnetite (Chemicell), mouse anti-ZO-1, and Alexa Fluor 488 goat anti-mouse, the live/dead cell viability assay (Invitrogen), Trypan Blue, 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), mounting medium, and mouse anti-cow glial fibrillary acidic protein (Dako) were used.

Cell Cultures. Human brain microvascular endothelial cells (HBMECs) were derived from an adult female diagnosed with epilepsy and immortalized by transfection with simian virus 40 large T antigen.[38] HBMECs were cultured in Medium 199 with L-glutamine and HEPES (Invitrogen) with 10% fetal calf serum (Invitrogen), 10% Nu Serum IV (BD Biosciences), and 100 units/mL penicillin G sodium and 100 μg/mL streptomycin sulfate (Invitrogen). Immortalized rat brain astrocytes (DI-TNC1) (ATCC) were cultured in DMEM/F12 (Lonza) with 10% fetal calf serum, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate.

Establishment of an *in Vitro* BBB Model in Transwell Membrane Plates. BCECs were seeded as monocultures in inserts of 12-well Transwell membrane culture plates at a density of 150000 cells/insert. The BCECs were cultured in astrocyte conditioned medium (ACM) consisting of a mixture of 50% astrocyte medium aspirated from astrocytes after incubation for 24 h and 50% HBMEC medium. When mentioned, astrocytes were seeded in the wells of the 12-well culture plates with 100000 cells/well. The astrocytes were grown overnight in a humidified incubator with 5% CO_2 to ensure proper cell attachment. Then the inserts containing BCECs were reinserted into the Transwell culture plates containing the astrocytes to form a noncontact coculture. The medium was replaced every day to avoid large changes in the pH of the medium.

Transendothelial Electrical Resistance (TEER) Measurements. TEER measurements were conducted with a Millicell ERS-2 apparatus (Millipore) and an STX-1 electrode (Millipore). To calculate the TEER, R_{blank} was subtracted from R_{sample} and the product multiplied by the well area. The Transwell insert in this study had a well area of 1.1 cm^2; therefore, the equation was as follows:

$$(R_{sample} - R_{blank}) \times 1.1\ cm^2 = \Omega\ cm^2$$

The TEER was measured every second day up until 7 days, and thereafter every day. Just before the TEER measurements were taken, the culture medium was changed and cells and medium were allowed to reach room temperature. Three measurements were taken on each well from which an average TEER value was calculated.

Fluorescent SPIONs. The SPIONs used in this study are commercially available magnetic iron oxide nanoparticles with a hydrodynamic diameter of 100 nm. They consist of a magnetic magnetite core surrounded by a lipophilic fluorescence dye covered by a hydrophilic polysaccharide matrix of starch consisting of α-D-glucose units. The magnetic core of the particles consists of approximately 101 single-domain magnetite crystallites of 12.3 nm each, and the intrinsic magnetization (M_s) of the particle is 350 kA/m.[39] Both red and blue fluorescent SPIONs were used in this study. The blue fluorescent nanoparticles have maximal excitation at 378 nm and emission at 413 nm, and red nanoparticles have an excitation wavelength of 578 nm and an emission wavelength of 613 nm.

Size and Charge of the SPIONs. The size [DLS/noninvasive back-scatter (NIBS)] equivalent to the particle diameter and charge/ζ potential were measured on a Zetasizer Nano instrument (Malvern). An aqueous solution containing 20 μg of SPIONs was diluted in 1 mL of doubly distilled water and tested in triplicate. The size of the SPIONs was analyzed on the basis of the cumulants method by the computer software that calculated the R_s values and provided the apparent size of the SPIONs. The ζ potential was likewise calculated by the software tested three times.

Application of SPIONs on the BBB Model. When the TEER of the BCECs reached a plateau, indicating that the highest TEER had been reached, and the endothelial cells had formed a barrier, the BCECs were removed from the original well plates. The fluorescent SPIONs were then added to the inserts in doses of 35, 70, and 140 μg/insert in three or four replicates at each concentration. The cells were then incubated with the SPIONs for 24 h, and afterward, the cells were washed three times with PBS to remove any excess SPIONs that had not been taken up by the BCECs and therefore potentially could pass the cell layer paracellularly. The inserts were then returned to the original well plates containing astrocytes. The experimental plates were

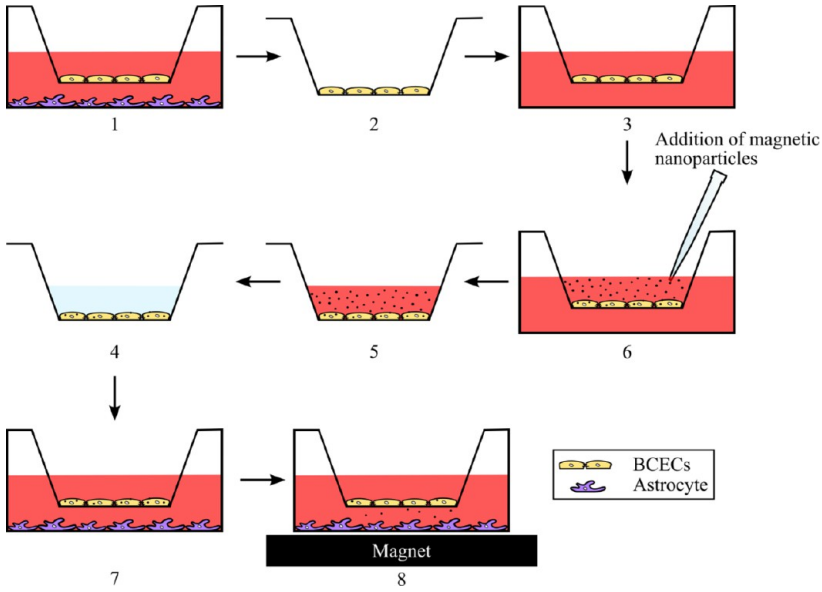

Figure 6. Drawing of the method employed for addition of SPIONs to the culture inserts with the aid of avoiding paracellular transport. SPIONs are depicted as blue dots. (1) The inserts were moved from well plates containing astrocytes cultured a remote distance from the endothelial cells (2) to empty well plates (3). After addition of the nanoparticles (4), the endothelial cells were incubated for 24 h. The medium from the inserts was then changed, and the inserts were washed three times with PBS to remove nanoparticles that had not been taken up by the BCECs (5 and 6). The inserts were supplied with fresh medium and were reinserted into the well plates containing astrocytes (7) and placed on a ferrite block magnet (black rectangle) for 5 h at 37 °C to draw the magnetic particles toward the bottom of the well (8) where astrocytes were cultured. A plate was also kept at 37 °C without being exposed to the magnet. After 5 h, the medium in the upper and lower chambers of the wells was collected and stored at 4 °C.

then subjected to a magnetic force for 5 h. The external magnetic force was applied by a ferrite block magnet (10.1 cm × 10.1 cm × 2.5 cm) with a field strength (B_r) of 0.39 T.

The force on a magnetic particle in a magnetic field can be determined by the equation $F_m = (\vec{\mu}\ \nabla)\vec{B}$, where μ is the magnetic moment of the particle. The description of the field and calculation of μ can be found in part 1 of the Supporting Information. The cell layer in this experiment was located 5 mm above the magnet surface, and the force on the magnetic particles was therefore approximately 2.5×10^{-21} N.

The process of addition of SPIONs to the cell culture and further performance of the experiments under the influence of a magnetic force are summarized in Figure 6.

The procedures described in Figure 6 were also performed on inserts that did not contain cells. This was done to investigate how many SPIONs would pass the membrane forming the bottom of the inserts after the washing step [Figure 6 (6)]. SPIONs were added to the cell free inserts in amounts of 35, 70, and 140 μg/insert in triplicate.

Immunostaining. After the experiment had been terminated, the cells were washed three times in PBS, fixed in 4% paraformaldehyde for 4 min, and washed three times in PBS. The cells were incubated overnight with mouse anti-zonula occludens-1 (ZO-1), and binding of the primary antibody was visualized using Alexa Flour 488 goat anti-mouse immunoglobulin. DI-TNC1s were incubated overnight with mouse anti-glial fibrillary acidic protein (GFAP), and binding of the primary antibody was visualized using Alexa Flour 488 goat anti-mouse immunoglobulin. The cell nuclei of both astrocytes and BCECs were stained with DAPI for 5 min. The membrane of the inserts containing BCECs was cut out of the insert, mounted on a slide with fluorescent mounting medium, and observed under an Axiovert 200 fluorescence microscope and LSM510-META laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany).

Cytotoxicity. To examine if the cells could be damaged by the SPIONs or by the application of the external magnetic field, we visualized the cell viability using a live−dead cell viability assay. The assay was performed according to the recommendations of the vendor. In brief, two working solutions were prepared: solution 1 containing 50 μM C12-resazurin in dimethyl sulfoxide (DMSO) and solution 2 consisting of 1 M SYTOX Green stain in DMSO. The culture medium was aspirated from the TW inserts, and 0.25 mL of PBS was added to each well. The working solutions were added to the wells to reach final concentrations of 5 μM C12-resazurin and 50 nM SYTOX Green dye in the two solutions. The cells where then incubated at 37 °C in an atmosphere of 5% CO_2 for 15 min and afterward were kept on ice, rinsed three times with PBS, and observed under a fluorescence microscope.

Dead and viable cells were counted on the basis of Trypan Blue labeling, as Trypan Blue enters only dead cells. Cells were cultured in monocultures in six-well culture plates until 100% confluence had been reached. Then SPIONs were added to half of the wells at a concentration of 1170 μg that corresponded to the largest dose (140 μg/insert) added in amount per square centimeter in the experiment described above. The cells were incubated with or without SPIONs for 24 h and placed on the plate magnet for 5 h. The cells were then trypsinized and mixed with Trypan Blue. An appropriate amount of cell suspension containing Trypan Blue was then filled in a hemocytometer, and dead and living cells were counted. The total amounts of dead and vital cells were calculated, and a Student's t test was performed to test if there were any differences in the amounts of vital and dead cells between the cells that had been or had not been subjected to the magnetic force. A p value of <0.05 was considered statistically significant.

Quantification of SPIONs Crossing the BBB *in Vitro*. The well plates in the presence of DI-TNC1 astrocytes were investigated under a fluorescence microscope with the medium remaining in the wells. The fluorescent SPIONs were counted using a counting mesh with an area of 0.054 mm^2 that was inserted inside the microscope's ocular. Counts were made at randomly picked areas 10 times per well to obtain a statistically correct counted average of the amount of nanoparticles in the wells. A standard curve was made by counts of different known concentrations of SPIONs. By using a Student's t test, we examined if there were any differences between the amounts of particles in the wells of the cells being subjected to the magnetic force and the amounts of particles in the wells of the cells that had not been subjected to the magnetic force. A p value of <0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

A mathematic delineation of how the magnetic forcs act on SPIONs in the *in vitro* experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Section of Neurobiology, Biomedicine, Department of Health Science and Technology, Fr. Bajers Vej 3B, Aalborg University, DK-9220 Aalborg East, Denmark. Phone: +45-99443731. E-mail: lbt@hst.aau.dk.

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Notes

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■ ABBREVIATIONS

BBB, blood–brain barrier; CNS, central nervous system; BCECs, brain capillary endothelial cells; DLS, dynamic light scattering; SPIONs, superparamagnetic iron oxide nanoparticles; TEER, transendothelial electric resistance; ZO-1, zonula occludens-1

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